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Identification of Specific Protein Markers that Correlate with Initiation and Progression of Microbially Influenced Corrosion

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Abstract

The subject of this report is the development of a screening method for detection of proteins in oil pipeline fluids and solids that could serve as indicators of microbially influenced corrosion (MIC). A method was developed that could detect proteins in sodium dodecyl sulfate (SDS) extracts of oil pipeline solids by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Procedures for sample preparation, electrophoresis, and staining for visualization are described. Results showed that different protein banding patterns could be observed in samples obtained from different locations. A change in banding pattern was also observed in samples collected from one area, 3 and 7 months later.

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I. Introduction

The objective of this research is to identify specific protein markers that correlate with the initiation and progression of microbially influenced corrosion (MIC). Economic losses resulting from damage and failure of structural materials due to corrosion have been estimated to be in the billions of dollars per year. Although it is unclear how much is due to MIC, it is reasonable to assume that it is significant. Current methods for identifying MIC involve chemical, electrochemical, metallurgical, and microbiological methods that are oftentimes expensive, laborious, and time consuming. Consequently, considerable damage has already occurred before MIC is detected. Identification of specific protein markers that signal the onset of or predisposition to MIC will facilitate early detection of MIC. Thus, timely institution of corrective measures will minimize economic losses due to corrosion.

II. Technical Objectives as outlined in the proposal

A. Develop a screening method for detection of proteins in oil pipeline fluids and solids that could serve as indicators of MIC.
B. Identify specific protein markers that correlate with MIC at various stages of progression.
C. Determine whether a specific pattern of appearance of protein markers correlate with extent or stage of corrosion.
D. Determine type of sample to be subjected to above analysis that will allow earliest possible detection of occurrence or onset of MIC.
E. Develop a method for quantifying identified protein markers and correlating it with extent or stage of corrosion.

III. Development of a screening method for detection of proteins in samples

This reporting period describes the development of a screening method (objective A) for detection of proteins in oil pipeline fluid and solid samples by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), a technique that separates proteins according to their molecular weights.

A. Sample preparation

Preliminary experiments using both produced water and pigging solid samples have indicated that pigging solids had more detectable protein than liquid samples. Centrifugation of 40 ml liquid samples at 17,000 x G did not yield a pellet with detectable protein. On the other hand, extraction of 1 to 2 g wet weight of pigging solids with 2 to 10 % SDS gave extracts with detectable protein bands following SDS-PAGE. Therefore, initial efforts concentrated on developing a reproducible methodology for detection of protein in pigging solids.

The pigging solids used in these experiments were collected by the major oil company who provided us with the samples during the period from April through November, 1990. During this time no biocide treatments were in effect. Piggings were simply run every 3 to 8 months. These samples were frozen as soon as possible after collection, shipped frozen and kept frozen until use. Large volumes were aliquoted into smaller portions for convenience and in order to avoid repeated freezing and thawing. The pigging solids contained varying amounts of water and oil. The protocol for sample preparation prior to electrophoresis that has yielded the best results so far is described as follows:

Approximately 2 grams wet weight of each sample were weighed out into a 25 ml glass Corex centrifuge tube with a teflon-lined screw cap. Hydrocarbon extraction was performed once with 15 ml of n-hexane with shaking in a wrist action shaker at room temperature for one hour. Hexane was removed following centrifugation at 8,000 x G for 15 minutes. Hydrocarbon extraction was repeated with 15 ml toluene under the same conditions as extraction with n-hexane. The solids were then washed with 10 ml of a 1:1 mixture of methanol and acetone with shaking at room temperature for 15 minutes. The methanol-acetone wash was removed following centrifugation at 8,000 x G for 15 minutes. The pellet was allowed to dry by evaporation at room temperature of any residual methanol-acetone under a fume hood. The solids were then extracted with 5 ml of 10% SDS in 0.05 M 2-[N-cyclohexylamino]-ethanesulfonic acid (CHES), pH 9.5 with shaking at room temperature overnight. The SDS extract was transferred to a 15 ml Corex centrifuge tube following centrifugation at 8,500 x G for 20 minutes and set aside. A second extraction with 3 ml of 10% SDS in 0.05 M CHES pH 9.5 was performed with shaking at room temperature for one hour. The SDS extracts were combined and any carried over particulates were removed by centrifugation at 8,500 x G. A 4 ml aliquot of the combined SDS extracts was transferred to a 15 ml Corex centrifuge tube and 2 ml of 60% (w/v) trichloroacetic acid (TCA) were added. The tube was kept in ice for one hour to allow precipitation of proteins in the extract. The tubes were centrifuged at 8,500 x G for 30 minutes. The pellets were

washed with ice cold 95 % ethanol. The washed pellets were redissolved in a minimum volume of 10% SDS in 0.05 M CHES pH 9.5. These were the samples used for SDS-PAGE.

B. SDS-PAGE

The procedure followed for SDS-PAGE was essentially that of Laemmli (3) using Bio-Rad's Mini Protean II electrophoresis system. Samples were diluted 1:1 with sample buffer and heated in a boiling water bath for 10 minutes. After cooling, 25 µl were applied to the gel using a Hamilton syringe. A 12 % separation gel was used. The sample and gel solutions used and the electrophoresis buffer composition are shown in Table 1. Electrophoresis was carried out at a constant voltage of 200 V for 40 minutes. Duplicate gels were usually run so that two methods of staining could be used.

C. Protein Staining

Two methods of staining for protein were used for visualization of protein bands following electrophoresis. Staining with Coomasie Brilliant Blue (CBB) G-250 was performed according to the method of Neuhoff et. al. (4). This method utilizes the colloidal properties of 0.1 % (w/v) CBB in 2% (w/v) phosphoric acid, 10% (w/v) ammonium sulfate, 20% (v/v) methanol which produces sensitive staining of proteins on a clear background. After electrophoresis, the gel is fixed in 12% (w/v) TCA for a minimum of one hour with gentle shaking. Fixing the gel overnight results in less background and more defined bands. The fixed gel is immersed in the staining solution for 2 to 4 hours with gentle shaking. After staining, the gel is briefly washed with 25 % (v/v) methanol. For prolonged storage, the gel was transferred to a solution of 20% (w/v) ammonium sulfate in water.

Silver staining was carried out using Bio-Rad's Silver Stain Plus Kit. The recommended procedure was followed with minor modifications. Extensive fixation (overnight) with two changes of the fixative solution and longer rinsing times in water were required to obtain good staining of protein bands with a minimum of background. In addition, staining time was reduced from 20 minutes to 5 minutes. Staining for 20 minutes resulted in a totally black gel.

D. Protein Assay

The method chosen for quantitative determination of protein in samples used for SDS-PAGE was the method of Smith, et. al. (5) using bicinchoninic acid purchased from Pierce Chemical Co. This method showed the least sensitivity to SDS. Presence of up to 1% SDS in the

sample did not interfere with the assay. However, only samples precipitable by TCA gave reliable results which were comparable to the results obtained on SDS-PAGE. Attempts to measure protein in SDS extracts prior to precipitation with TCA grossly overestimated the protein concentration in the extracts. Similar results were obtained using the Bradford (1) method which was more sensitive to SDS interference.

IV. Results and Discussion

The procedure described above gave reasonably good results following SDS-PAGE. A number of pigging solids (Table 2) were analyzed according to the above procedure. The protein concentrations of the TCA-precipitated extracts which were used for electrophoresis are shown in Table 3. Photographs of the resulting gels stained with either CBB G-250 or silver are shown in Figures 1-4. These results show that different protein banding patterns can be observed in samples obtained from different locations. For example, in Figs. 1 and 2, lane 5, a sample collected from the paddle of a gas flotation cell that removes oil and solids from produced water, shows a markedly different pattern from lanes 2,3, and 4, which were pigging solids from drill site (DS) 11 (a manifold building for gathering well line flows into common lines), pigged solids from the line connecting IMF2 (an intermediate manifold building for seawater) to DS 11, and pigging solids from the produced water injection line of DS 4, respectively. Lanes 2 and 3 in Fig. 1, a gel stained with CBB G-250, appear to be identical and very similar to lane 4. However, subtle differences in the relative intensities of the protein bands in each sample as compared to Fig. 1, can be observed in Fig. 2, a duplicate gel stained with silver.

Figures 3 and 4 are again duplicate gels stained with CBB G-250 and silver, respectively. Again different banding patterns can be observed from samples collected from different locations. The most interesting information that can be obtained from these gels are manifested in lanes 3, 7, and 8. These samples were pigging solids from the produced water injection line of DS 13 but were collected on different dates. Lanes 7 and 8 (samples collected on 7/29/90 and 11/25/90, respectively) show a major band (identified by an arrow in Fig. 3) which is not present in lane 3 (sample collected on 4/10/90). This band stains yellow in the duplicate silver stained gel (Fig. 4). It is possible that this protein band consists of lipo- and/or sialo-glycoproteins which have been shown to stain yellow with silver (2). At this time we are not certain whether this result is a consequence of the extraction procedure or whether it is signalling a change in the microbial population prevailing in the area. This will be investigated during the next reporting period, when we commence running 2-dimensional gels on these samples. Procedures will also be optimized and standardized to obtain reproducible results.

The above results need to be correlated with the corrosion status of the sampling areas at the time the samples were collected. We are in the process of obtaining this information from the oil company which provided us with these samples. In addition, we have made arrangements to obtain more recent samples for analysis and correlation with corrosion status.

In conclusion, we have successfully met Objective A within the time frame set forth in the proposal: development of a screening method for detection of proteins in oil pipeline fluids and solids that could serve as indicators of MIC, within one month following initiation of the project. More samples will be screened and analyzed to accomplish Objectives B-E within the next 5 months.

V. Literature Cited

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2. Deh, M. E., J. K. Dzandu, and G. E. Wise (1985) Anal. Biochem. 150, 166-173.

3. Laemmli, U. K. (1970) Nature 227, 680-685.

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Table 1

Separation Gel, Sample, and Electrophoresis Buffer Solutions

12% Separation Gel

Stock Solution	Volume
Distilled water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.50 ml
10% (w/v) SDS	100 µl
Acrylamide/Bis (30%)	4.00 ml
10% (w/v) ammonium persulfate	50 µl
Total volume	10.0 ml

Sample Buffer (SDS reducing buffer)

Stock Solution	Volume
Distilled water	3.2 ml
0.5 M Tris-HCl, pH6.8	1.0 ml
Glycerol	1.6 ml
10% (w/v) SDS	1.6 ml
ß-mercaptoethanol	0.4 ml
0.1% (w/v) Bromophenol Blue	0.2 ml
Total volume	8.0 ml

Electrophoresis Buffer Composition, pH 8.3

Tris base	3.0 g/L
Glycine	14.4 g/L
SDS	1.0 g/L

Table 2

List of Solid Samples Analyzed by SDS-PAGE

Sample #	Date Collected	Sampling Location or Area
1	4/3/90, 9:43	Drill Site (DS) 11 pig solids
2	4/3/90, 9:42	IMF2-DS 11 pig solids
3	4/7/90	IMF2-DS 11 pig receiver solids
13	5/1/90	DS 4 produced water injection (PWI) pig solids
14	5/1/90, 11:11	DS 4 PWI pig solids
15	5/1/90	DS 4 PWI pig trap solids
7	4/10/90	DS 13 PWI pig solids
103	7/29/90	DS 13 PWI pig trap solids
160	11/25/90	DS 13 PWI pig trap solids
73	6/9/90	DS 9 PWI pig trap solids
99	8/2/90	FS2 (Flow Station 2) GFC1 paddle solids

FS 2 GFC1 paddle, the paddle off of a gas flotation cell inside Flow Station 2

Table 3

Protein Concentrations of Extracts from Solid Samples Used for SDS-PAGE

Sample #	Date Collected	Sampling Location or Area	Protein (mg/ml)
1	4/3/90	DS 11 pig solids	0.42
2	4/3/90	IMF2-DS 11 pig solids	0.70
3	4/7/90	IMF2-DS 11 pig receiver solids	0.36
13	5/1/90	DS 4 PWI pig solids	0.42
14	5/1/90	DS 4 PWI pig solids	0.47
15	5/1/90	DS 4 PWI pig trap solids	0.47
7	4/10/90	DS 13 PWI pig solids	1.73
103	7/29/90	DS 13 PWI pig trap solids	1.42
160	11/25/90	DS 13 PWI pig trap solids	1.45
73	6/9/90	DS 9 PWI pig trap solids	0.20
99	8/2/90	FS 2 GFC1 paddle solids	1.12

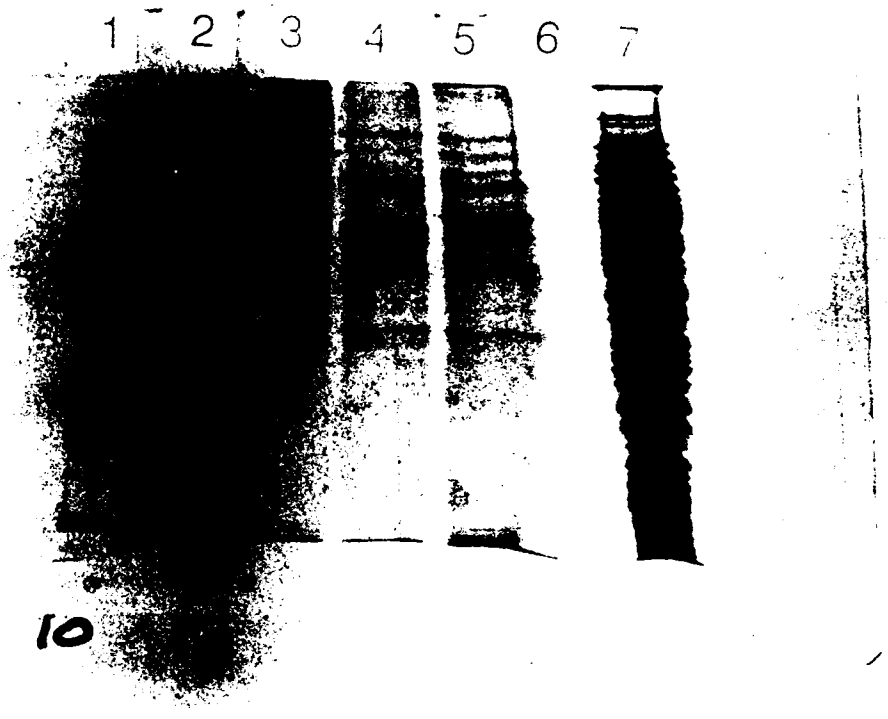

Fig. 1. SDS-PAGE of solid samples stained with CBB G-250. Lane 1, MW standards (97.4, 66.2, 45.0, 31.0, 21. 5, 14.4 Kd); lane 2, sample #1, DS 11 pig solids, 4/3/90 (5.2 μg); lane 3, sample #2, IMF2-DS 11 pig solids, 4/3/90 (8.8μg); lane 4, sample #14, DS 4 PWI pig solids, 5/1/90 (5.9 μg); lane 5, sample #99, FS 2 GFC1 paddle solids, 8/2/90 (14 μg); lane 7, E. coli cell lysate (66 μg).

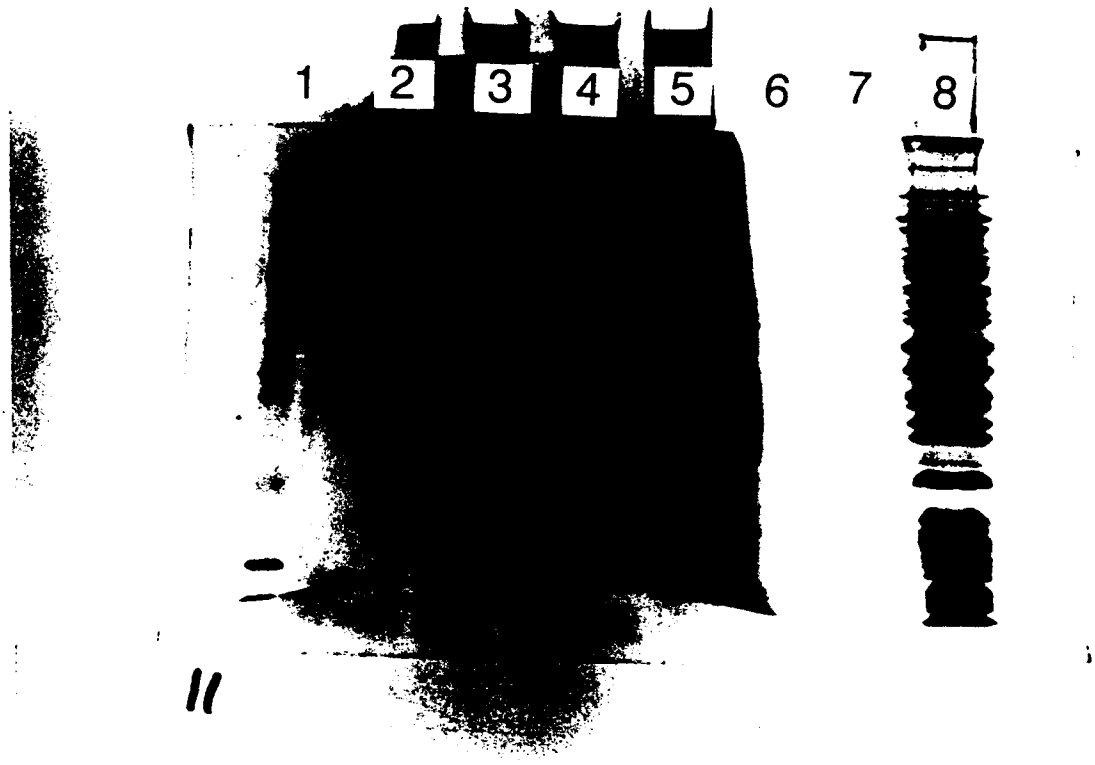

Fig. 2. SDS-PAGE of solid samples stained with silver. Lanes 1-5, same samples as in Fig. 1. E. coli lysate was loaded in lane 8.

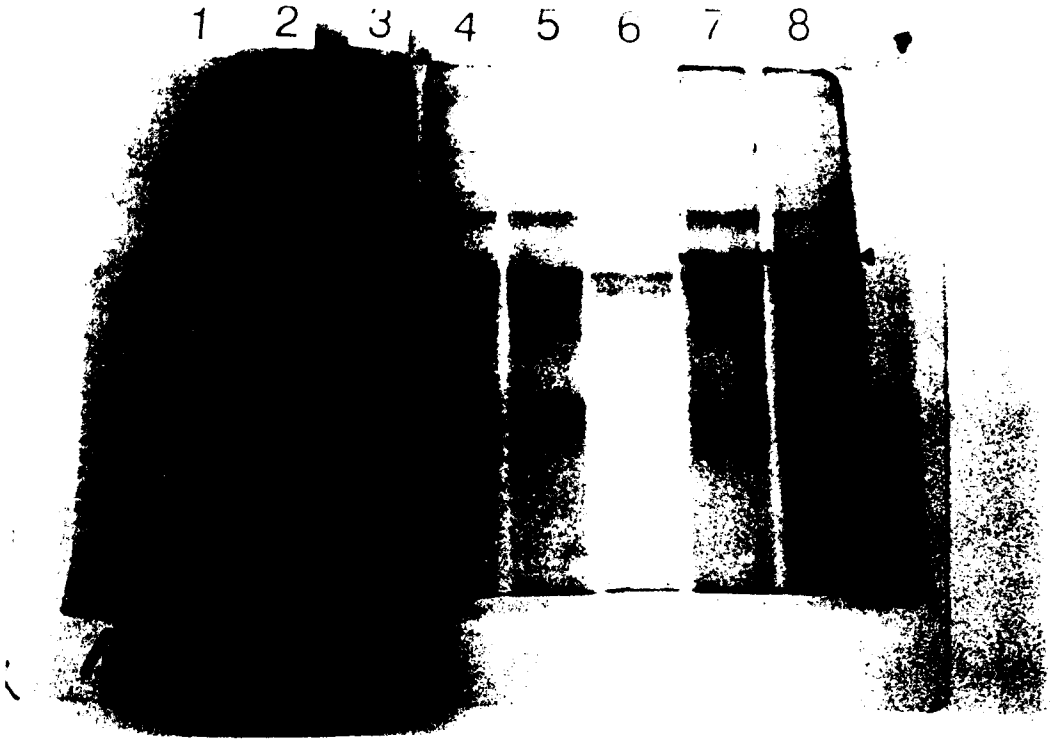

Fig. 3. SDS-PAGE of solid samples stained with CBB G-250. Lane 1, MW standards (an old preparation was used, showing deterioration of the protein bands); lane 2, sample #3, IMF2-DS 11 pig receiver solids, 4/7/90 (4.5 μg); lane 3, sample #7, DS 13 PWI pig solids, 4/10/90 (22 μg); lane 4, sample #13, DS 4 PWI pig solids, 5/1/90 (5.2 μg); lane 5, sample #15, DS 4 PWI pig trap solids, 5/1/90 (5.9 μg); lane 6, sample #73, DS 9 PWI pig trap solids, 6/9/90 (2.5 μg); lane 7, sample #103, DS 13 PWI pig trap solids, 7/19/90 (18 μg); lane 8, sample #160, DS 13 PWI pig trap solids, 11/25/90 (18 μg); (note arrow on additional band in lanes 7 and 8 compared to sample in lane 3).

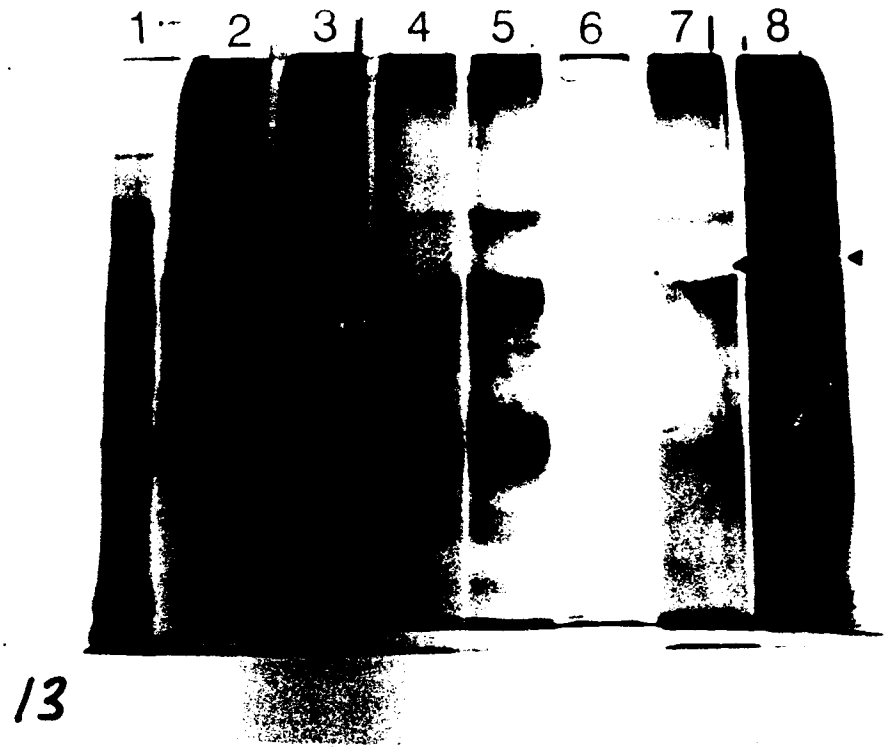

Fig. 4. SDS-PAGE of solid samples stained with silver. The same samples in each lane as in Fig. 3 (note arrow on additional band in lanes 7 and 8, compared to sample in lane 3).